

Exhibit 1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Goddard, et al.
Appl. No.	: 10/063,546
Filed	: May 2, 2002
For	: ANTIBODIES TO A POLYPEPTIDE ENCODED BY A NUCLEIC ACID OVEREXPRESSED IN NORMAL STOMACH, NORMAL SKIN AND KIDNEY TUMOR
Examiner	: Patricia Ann Duffy
Group Art Unit	: 1645

DECLARATION UNDER 37 CFR §1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

We declare and state as follows:

1. We are inventors of the invention claimed in the above-captioned patent application.
2. During the time period in which we participated in the events and activities described herein, we were employed by Genentech, Inc., the assignee of the above-captioned application.
3. All of the events and activities described herein were performed in the U.S. by one or more of us personally, or by others at the direction of a co-inventor, as part of our duties as employees of Genentech, Inc.
4. We understand that the PTO has rejected claims of this application as lacking novelty in view of Barnes (WO 00/18904), which published April 6, 2000. We understand that the PTO asserts that Barnes discloses a protein, termed TANGO 215 and listed as SEQ ID NO:32 in Barnes, possessing an amino acid sequence that is 91.3% identical to SEQ ID NO:38 (PRO1344) of the above-captioned application. We understand that the PTO also asserts that Barnes also discloses generically antibodies directed to TANGO proteins.

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6. Exhibit A demonstrates that, no later than August 18, 1998, I and/or my co-inventors were in possession of as much of the claimed invention as the PTO asserts is disclosed in Barnes.

7. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

By: Audrey Goddard Date: 27 Mar 02
Audrey Goddard

By: _____ Date: _____
Paul J. Godowski

By: _____ Date: _____
Austin L. Gurney

By: _____ Date: _____
William I. Wood

By: _____ Date: _____
Christopher J. Grimaldi

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By: _____

Date: _____

Audrey Goddard

By: _____

Date: 09/11/08

Paul J. Godowski

By: _____

Date: _____

Austin L. Gurney

By: _____

Date: _____

William I. Wood

By: _____

Date: _____

Christopher J. Grimaldi

4801507
012408

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By: _____ Date: _____
Audrey Goddard

By: _____ Date: _____
Paul J. G. Nowski

By:  _____ Date: 3/7/08
Justin L. Gurney

By: _____ Date: _____
William I. Wood

By: _____ Date: _____
Christopher J. Grimaldi

4801307
012408

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Audrey Goddard

Date: _____

By: _____
Paul J. Godowski

Date: _____

By: _____
Austin L. Gurney

Date: _____

By: William I. Wood
William I. Wood

Date: 3/16/02

By: _____
Christopher J. Grimaldi

Date: _____

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Date: _____

By: _____
William I. Wood

Date: _____

By: _____
Christopher J. Grimaldi

Date: 3/11/08

4801507
012408

Exhibit 2



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/063,545	05/02/2002	Dan L. Eaton	P3230R1C001-168	1059
20995 7590 12/26/2006 KNOBBE MARTENS OLSON & BEAR LLP 2040 MAIN STREET FOURTEENTH FLOOR IRVINE, CA 92614			EXAMINER DUFFY, PATRICIA ANN	
			ART UNIT	PAPER NUMBER
			1645	
SHORTENED STATUTORY PERIOD OF RESPONSE		NOTIFICATION DATE		DELIVERY MODE
3 MONTHS		12/26/2006		ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

If NO period for reply is specified above, the maximum statutory period will apply and will expire 6 MONTHS from the mailing date of this communication.

Notice of this Office communication was sent electronically on the above-indicated "Notification Date" and has a shortened statutory period for reply of 3 MONTHS from 12/26/2006.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

jcartee@kmob.com
eOAPilot@kmob.com

Office Action Summary	Application No.		Applicant(s)	
	10/063,545		EATON ET AL.	
	Examiner		Art Unit	
	Patricia A. Duffy		1645	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --
Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
 - If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
 - Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED. (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 10 October 2006.
- 2a) ☒ This action is FINAL. 2b) ☐ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 6-8, 11-17 is/are pending in the application.
- 4a) Of the above claim(s) _____ is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 6, 8 and 12-17 is/are rejected.
- 7) ☒ Claim(s) 7 and 11 is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. _____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|---|--|
| <p>1) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892)</p> <p>2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948)</p> <p>3) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08)
 Paper No(s)/Mail Date <u>2006</u></p> | <p>4) <input type="checkbox"/> Interview Summary (PTO-413)
 Paper No(s)/Mail Date _____</p> <p>5) <input type="checkbox"/> Notice of Informal Patent Application</p> <p>6) <input type="checkbox"/> Other: _____</p> |
|---|--|

RESPONSE TO AMENDMENT

The amendment, response and declarations filed 10-10-06 has been entered into the record. Claims 1-5 and 9-10 have been cancelled. Claims 6-8 and 11-17 are pending and under examination.

The text of Title 35 of the U.S. Code not reiterated herein can be found in the previous office action.

Rejections Withdrawn

Claims 6-8 and 11-17 are rejected under 35 U.S.C. 101 because the claimed invention lacks patentable utility due to its not being supported by a specific, substantial and credible utility or, in the alternative a well-established utility is withdrawn for reasons set forth below.

Claims 6-8 and 11-17 are also rejected under 35 U.S.C. 112, first paragraph. Specifically, since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention is withdrawn for reasons set forth below.

Applicants' response states that the gene expression data in the specification, Example 18, shows that the mRNA associated with the polypeptide was more highly expressed in kidney tumor tissue as compared to normal kidney or more highly expressed in normal stomach and skin as compared to stomach or melanoma tumors. Gene expression was analyzed using standard semi-quantitative PCR amplification reactions of cDNA libraries isolated from different human tumor and normal human tissue samples. Identification of the differential expression of the polypeptide-encoding gene in tumor tissue compared to the corresponding normal tissue renders the molecule per se and antibodies that specifically bind the molecule useful and enabled as a diagnostic tool for the determination of the presence or tumor.

Example 18 at page 140 of the instant specification demonstrates differential expression of DNA58723-1588 cDNA using qualitative PCR amplification reactions. DNA58723-1588 was shown to be more highly expressed in esophageal and kidney tumors as compared to the corresponding normal tissue samples in this Example. Applicant states in the response that Example 18 utilizes a more accurate and reliable method of assessing changes in mRNA levels, namely quantitative PCR analysis. Applicant relies on more than 100 references, where expression levels of mRNA, measured by quantitative PCR, were found to have a good correlation to the expressed protein levels.

It had been previously argued in the office actions of record that mRNA levels were not predictive of protein levels, citing several references including Haynes et al, Gygi et al and Chen et al. However, these references were measuring and analyzing mRNA levels using microarrays, not using quantitative PCR analysis and the art recognizes the results obtained by microarray are not always the same as the results obtained using quantitative PCR (for example see Oda et al. *Virchows. Arch.* 430:99-105, 1997, specifically page 104, column 1, paragraph 2). While the PTO found several references in which the protein expression levels did not correlate with mRNA levels measured by quantitative PCR (see Sugg et al, *Clinical Endocrinology* 49:629-637, 1998; Toler et al. *Am. J. Obstet. Gynecol.* 194:e27-231, 2006; Berner et al *Histopathol* 42:546-554, 2003; Brooks et al *Am. J. Renal Physiol* 284:F218-F228, 2003), the majority of the references which were found, including those cited by Applicant, demonstrated a correlation between mRNA levels measured by quantitative PCR and protein expression levels.

Applicant asserts that the expression levels of protein correlate to mRNA (cDNA) levels when the cDNA is measured by quantitative PCR (i.e. rt-PCR). Applicant has provided more than 100 references in support of this position. The prior art of record (Haynes et al, Gygi et al, Chen et al.) argued by the Examiner, is not specifically directed to message levels measured by rt-PCR. Based on the totality of evidence of record, one of skill in the art would find it more likely than not that an increase in message as measured

by rt-PCR would be predictive of an increase in protein expression levels, absence evidence to the contrary. Therefore, the data presented in Example 18, which demonstrates differential expression of nucleic acids encoding the polypeptide, also supports a conclusion of differential expression of the polypeptide. Therefore, one of ordinary skill in the art would be able to use the antibodies that specifically bind the polypeptide diagnostically for distinguishing tumor from normal tissue as asserted by Applicant.

Rejections Maintained

Claims 14-17 stand rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention is maintained for reasons made of record in the office Action mailed 3-29-05, 1-5-06 and *supra*.

This rejection pertains to the issue of percent identity. Applicants again argue the issue of combination of structure with function. This is again not persuasive because the "generation of an antibody that specifically detects the polypeptide of SEQ ID NO:38 " is not a function of the polypeptide per se. Applicants argue that immunological function is a function of the polypeptide. This is not persuasive because the polypeptide has no function in regulating the immune response. It is not a cytokine or receptor that is immunoregulatory. It has no immune function. The ability to mount an immune response is not a function of the antigen, but a function of the hosts' ability to respond. As such, the ability to raise an antibody is not a function of the polypeptide, but a function of the host in which the antibody is raised. Applicants again argue Wallach. Wallach does not speak to variants of a polypeptide but nucleic acids encoding the same polypeptide. The correlation of the structure of the nucleic acid with the structure is predictable given the Wobble hypothesis. Therefore, a genus of nucleic acids encoding the same polypeptide was

described. This is not the instant fact scenario. The instant case the polypeptides are different and encoded by different nucleic acids. Applicants argue that there is nothing in Example 14 of the written description guidelines that require that the function limit the structure of the variant protein in any discernable, predictable or disclose manner. This is not persuasive because the claimed function is not a function of the protein for all the reasons made of record. Applicants argue that there is nothing about the claims that require that the antibodies generated by the variant polypeptides do not bind themselves in addition to SEQ ID NO:38. If this is so.. what does the term "specifically detect SEQ ID NO:38 mean"? Specificity is defined in The Dictionary of Immunology as "A term defining *selective reactivity* [emphasis added] between substances, e.g. of an antigen with its corresponding antibody or primed lymphocyte." (Herbert et al eds, The Dictionary of Immunology, Fourth Edition, Academic Press, 1995, page 147). Here, Applicants claims require that the "selectivity" not be toward the variant polypeptide that provokes the immune response in a host, but a different polypeptide. This usage of specifically binds is contrary to Applicants own use of "specific" in the specification as it relates to antibodies at [0366] which teaches that "... is typically immunized with an immunizing antigen to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent.". Immunological activity is set forth in the specification as the ability to induce the production of an antibody against an antigen epitope possessed by a native or naturally-occurring PRO. This paragraph in context with [0366] conveys to the skilled artisan the ability to raise "specifically binding antibodies" against itself, when itself is used as an immunogen and not variant peptides. The specification as filed does not describe a variant polypeptide that generates an antibody that specifically binds a different polypeptide. The specification does not describe variants with the claimed property. The genus encompasses antibodies that specifically detect SEQ ID NO:38 polypeptide wherein the immunizing polypeptides have numerous differences in amino acid sequences, including numerous differences in linear and conformational epitopes. However,

the present specification fails to provide sufficient disclosure of such polypeptides that maintain the structural and functional properties of the claimed limitation of specifically detecting SEQ ID NO:38 in stomach, skin or kidney tissue samples. The specification does not provide sufficient guidance as to which of the amino acids may be changed while 'claimed antigen specificity' structural or functional activity and specificity is retained. Furthermore, Lederman et al. (Molecular Immunology 28: 1171-1181, 1991) disclose that a single amino acid substitution in a common allele ablates binding of a monoclonal antibody (see entire document) and Li et al. (PNAS 77: 3211-3214, 1980) disclose that dissociation of immunoreactivity from other biological activities when constructing analogs (see entire document). As such, in the absence of the description of a representative number of species of polypeptides that fall within the genus and have the recited property, the skilled artisan would readily appreciate that the disclosure of a single SEQ ID NO did not place Applicants in possession of the now claimed genus at the time of filing.

Claims 6, 8 and 12-17 stand rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention is maintained for reasons made of record in the office action mailed 1-5-06.

Applicants argues that the standard for definiteness does not require that the particular structure of the signal sequence be defined in the claims because the claims are read in light of the specification. This argument is not persuasive, the claim read in light of the specification at page 119, paragraph [0441] teach that various polypeptide-encoding nucleic acid sequences were identified by applying a **proprietary** signal sequence finding algorithm developed by Genetech, Inc.. The algorithm is not described in the specification. The skilled artisan would not be readily apprised of the specifics used to determine signal sequences. This passage does not provide the metes and bounds of any signal polypeptide *per se*. In fact, a review of the figures indicates that the signal

sequences disclosed are different lengths and structures and therefore, in the absence of a precise delineation in the claims, the metes and bounds of any signal sequence is insolubly indefinite, especially in that a admittedly proprietary algorithm was used to identify such. The skilled artisan in this art is not readily apprised of admitted proprietary information and algorithms. As such, the metes and bounds of the "signal sequence" is *prima facie* indefinite and limitations from the specification or figure are not read into the claims.

Claims 14-17 stand rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention for reasons made of record in the office action mailed 1-5-06 and herein.

This rejection pertains to the issue of new matter in view of the wherein phrase of the independent claims 14 and 15. Applicants argue that cross-reactive antibodies are contemplated by the specification as filed. This is not persuasive, the relied upon passage does not provide for the concept in the claims. Applicants argue that there is nothing about the claims that require that the antibodies generated by the variant polypeptides do not bind themselves in addition to SEQ ID NO:38. If this is so, what does the term "specifically detect SEQ ID NO:38 mean"? Specificity is defined in The Dictionary of Immunology as "A term defining *selective reactivity* [emphasis added] between substances, e.g. of an antigen with its corresponding antibody or primed lymphocyte." (Herbert et al eds, The Dictionary of Immunology, Fourth Edition, Academic Press, 1995, page 147). Here, Applicants claims require that the "selectivity" not be toward the variant polypeptide that provokes the immune response in a host, but a different polypeptide. This usage of specifically binds is contrary to Applicants own use of

"specific" in the specification as it relates to antibodies at [0366] which teaches that "... is typically immunized with an immunizing antigen to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent.". Immunological activity is set forth in the specification as the ability to induce the production of an antibody against an antigen epitope possessed by a native or naturally-occurring PRO. This paragraph in context with [0366] conveys to the skilled artisan the ability to raise "specifically binding antibodies" against itself, when itself is used as an immunogen and not variant peptides.

Status of Claims

All claims stand rejected. Claims 7 and 11 are objected to as depending from a rejected base claim.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Patricia A. Duffy whose telephone number is 571-272-0855. The examiner can generally be reached on M-Th 6:30 am - 6:00 pm. If attempts to reach the examiner by telephone are unsuccessful, the examiner's Supervisor, Jeffrey Siew can be reached on 571-272-0787.

The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Patricia A. Duffy
Patricia A. Duffy

Primary Examiner
Art Unit 1645

Notice of References Cited	Application/Control No. 10/063,545	Applicant(s)/Patent Under Reexamination EATON ET AL.	
	Examiner Patricia A. Duffy	Art Unit 1645	Page 1 of 1

U.S. PATENT DOCUMENTS

*	Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
A	US-			
B	US-			
C	US-			
D	US-			
E	US-			
F	US-			
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H	US-			
I	US-			
J	US-			
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FOREIGN PATENT DOCUMENTS

*	Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
N					
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NON-PATENT DOCUMENTS

*	Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
U	Lederman et al. (Molecular Immunology 28: 1171-1181, 1991)
V	Li et al. (PNAS 77: 3211-3214, 1980)
W	Herbert et al eds, The Dictionary of Immunology, Fourth Edition, Academic Press, 1995, page 147
X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Exhibit 3

A SINGLE AMINO ACID SUBSTITUTION IN A COMMON AFRICAN ALLELE OF THE CD4 MOLECULE ABOLISHES BINDING OF THE MONOCLONAL ANTIBODY, OKT4

SETH LEDERMAN,*† JULIE A. DeMARTINO,‡ BRUCE L. DAUGHERTY,‡ IVAN FOELDVAR,†
MICHAEL J. YELLIN,* AILEEN M. CLEARY,* NOAH BERKOWITZ,* ISRAEL LOWY,*
NED S. BRAUNSTEIN,* GEORGE E. MARK‡ and LEONARD CHESSE*

*Department of Medicine, Columbia University, New York, NY 10032, U.S.A.; and the †Department of Cellular and Molecular Biology, Merck, Sharp & Dohme Research Laboratories, Rahway, NJ 07065, U.S.A.

(First received 12 December 1990; accepted in revised form 2 January 1991)

Abstract—The CD4 molecule is a relatively non-polymorphic 55 kDa glycoprotein expressed on a subset of T lymphocytes. A common African allele of CD4 has been identified by non-reactivity with the monoclonal antibody, OKT4. The genetic basis for the OKT4⁺ polymorphism of CD4 is unknown. In the present paper, the structure of the CD4 molecule from an homozygous CD4^{OKT4⁺} individual was characterized at the molecular level. The size of the CD4^{OKT4⁺} protein and mRNA were indistinguishable from those of the OKT4⁺ allele. The polymerase chain reaction (PCR) was used to map the structure of CD4^{OKT4⁺} cDNAs by amplifying overlapping DNA segments and to obtain partial nucleotide sequence after asymmetric amplification. PCR was then used to clone CD4^{OKT4⁺} cDNAs spanning the coding region of the entire, mature CD4 protein by amplification of two overlapping segments followed by PCR recombination. The nucleotide sequence of CD4^{OKT4⁺} cDNA clones revealed a G→A transition at bp 867 encoding an arginine→tryptophan substitution at amino acid 240 relative to CD4^{OKT4⁺}. Expression of a CD4^{OKT4⁺} cDNA containing only this transition, confirmed that the arginine→tryptophan substitution at amino acid 240 abolishes the binding of the mAb OKT4. A positively charged amino acid residue at this position is found in chimpanzee, rhesus macaque, mouse and rat CD4 suggesting that this mutation may confer unique functional properties to the CD4^{OKT4⁺} protein.

INTRODUCTION

The CD4 molecule is a relatively non-polymorphic, 55 kDa surface glycoprotein comprised of four extracellular domains, a hydrophobic transmembrane region and a hydrophilic cytoplasmic tail (Maddon *et al.*, 1985, 1987). The NH₂-terminal extracellular domain (V1) has striking sequence homology to immunoglobulin (Ig) light chain variable regions (Maddon *et al.*, 1985). The other extracellular domains (V2–V4) are homologous to other members of the Ig gene superfamily. For example, the V3 domain is related by amino acid sequence and predicted secondary structure to the poly Ig receptor (Clark *et al.*, 1987). The CD4 molecule is expressed predominantly on the cell membrane of helper T lymphocyte subsets which recognize peptide antigens bound to Class II MHC (Ia) molecules (White *et al.*, 1978; Reinherz and Schlossman, 1980; Thomas *et al.*, 1983; Dyalnas *et al.*, 1983; Janeway *et al.*, 1988).

On the cell surface, CD4 expression mediates binding to Ia molecules (Doyle and Strominger, 1987) as

well as functional interactions of helper and cytotoxic CD4⁺ T cells with Ia bearing targets (Webb *et al.*, 1979; Biddison *et al.*, 1982; Krensky *et al.*, 1982; Meurer *et al.*, 1982; Rogozinski *et al.*, 1984; Gay *et al.*, 1987). Mutagenesis and epitope mapping by antibodies, have demonstrated that the two most NH₂-terminal domains of CD4 (V1 and V2) contribute to the Ia binding structure of CD4 (Clayton *et al.*, 1989; Lamarre *et al.*, 1989). The NH₂-terminal domains of CD4 also contain the binding residues most important in the interaction of CD4 with gp120, the envelope glycoprotein of human immunodeficiency virus (HIV) (Jameson *et al.*, 1988; Richardson *et al.*, 1988; Peterson and Seed, 1988; Landau *et al.*, 1988; Clayton *et al.*, 1988; Arthos *et al.*, 1989). It is the relatively high affinity interaction of the CD4 V1 domain with gp120 that renders CD4 the primary receptor for HIV entry into human cells (McDougal *et al.*, 1986a,b; Lifson *et al.*, 1986; Lasky *et al.*, 1986; Smith *et al.*, 1987; Fisher *et al.*, 1988; Deen *et al.*, 1988; Trautnecker *et al.*, 1988; Hussey *et al.*, 1988). Although CD4-Ia and CD4-gp120 binding mediate cell-cell adhesion, CD4 molecules also participate in signaling events that regulate T cell activation (Bank and Chess, 1985; Moldwin *et al.*, 1987; Carrel *et al.*, 1988; Janeway, 1989). In this regard, CD4 is physically associated with a protein tyrosine kinase,

† Author to whom correspondence should be addressed at: Black Building 808, Columbia University, 630 W. 168th St., New York, NY 10032, U.S.A.

p56^{lck} and it is thought that the interaction of p56^{lck} with the cytoplasmic tail of CD4 is important in signaling (Turner *et al.*, 1990).

The functional significance of the two COOH-terminal domains of CD4 (V3 and V4) has been less well studied. The monoclonal antibody (mAb) OKT4 recognizes a trypsin resistant, membrane proximal portion of the V3 domain of the CD4 molecule (Rao *et al.*, 1983). Because OKT4 does not inhibit interactions of CD4 with Ia (Rogozinski *et al.*, 1984) or gp120 (McDougal *et al.*, 1985, 1986a) and does not induce signals to T cells (Bank and Chess, 1985), it is not known if the V3 domain participates in distinct functions. Individuals have been identified whose CD4 molecules bind a variety of mAbs to the V1 and V2 domains of CD4 but do not bind OKT4 (Bach *et al.*, 1981; Fuller *et al.*, 1984; Karol *et al.*, 1984; Aozasa *et al.*, 1985; Sato *et al.*, 1984; Stohl and Kunkel, 1984). The expression of the OKT4⁺ phenotype has been determined by family studies to be inherited in an autosomal codominant fashion (Bach *et al.*, 1981; Fuller *et al.*, 1984; Karol *et al.*, 1984; Aozasa *et al.*, 1985; Sato *et al.*, 1984; Stohl and Kunkel, 1984). The allele is common in people of African descent, but rare in people of other racial origins (Fuller *et al.*, 1984; Aozasa *et al.*, 1985; Stohl and Kunkel, 1984). Specifically, 8.2% of African-Americans are homozygous and 20.2% heterozygous for this OKT4⁺ phenotype (Fuller *et al.*, 1984). In contrast, less than 0.43% of Japanese subjects are homozygous OKT4⁺ (Aozasa *et al.*, 1985). The OKT4⁺ allele has not been reported in Caucasians (Fuller *et al.*, 1984; Stohl and Kunkel, 1984). At the present time the structural or genetic basis for this polymorphism in the CD4 molecule is unknown.

In addition, the functional consequences of the OKT4⁺ phenotype are not completely understood. Cells expressing CD4 molecules that are OKT4⁺ are susceptible to HIV infection both *in vitro* and *in vivo* (Hoxie *et al.*, 1986). In general, OKT4⁺ individuals appear to have grossly normal immune functions and are not immunosuppressed (Bach *et al.*, 1981; Fuller *et al.*, 1984; Karol *et al.*, 1984; Aozasa *et al.*, 1985; Sato *et al.*, 1984; Stohl and Kunkel, 1984). The OKT4⁺ phenotype may be associated with the autoimmune disease, systemic lupus erythematosus (SLE) (Ichikawa *et al.*, 1983; Stohl and Singer, 1984; Stohl *et al.*, 1985). It has been shown that homozygous OKT4⁺ individuals with SLE, have a functional defect in T-dependent, B cell differentiation (Stohl *et al.*, 1985). As importantly, this functional defect is observed in the unaffected (non-SLE), OKT4⁺ siblings of OKT4⁺ SLE patients (Stohl *et al.*, 1985). In order to elucidate the structural basis for the OKT4⁺ phenotype and to further define functions associated with this polymorphism of the CD4 molecule, the present study identified the amino acid substitution in CD4 that confers the OKT4⁺ phenotype.

MATERIALS AND METHODS

Cell culture

The Jurkat and Epstein-Barr virus (EBV) secreting, marmoset cell lines [American Type Culture Collection (ATCC), Rockville, MD] as well as B lymphoblastoid cells (see below) were cultured in IMDM supplemented with 10% FCS. The 293 cell line (ATCC) was cultured in DMEM supplemented with 10% FCS, glutamine and essential amino acids.

Monoclonal antibodies

The mAbs, OKT4, OKT8 and W6/32 were produced by hybridomas available from the American Type Culture Collection (Rockville, MD) and purified from ascites fluid on protein A columns (Biorad, Rockville Center, NY). The mAb OKT4A was purchased from Ortho Pharmaceutical Division (Raritan, NJ).

Cytofluorographic analysis

Approximately 10⁶ cells were incubated with 100 ng of the indicated mAb for 45 min at 4°C. Cells were washed to remove unbound mAb before incubation with goat anti-mouse Ig secondary antibody coupled to fluorescein (Cappel, Cochranville, PA) and fluorescence intensity was measured on a FACSCAN Cytofluorograph (Becton-Dickinson, Mountainview, CA).

Generation of CD4⁺ B lymphoblastoid cell lines

Peripheral blood lymphocytes were obtained from the freshly drawn blood of volunteers by centrifugation of Ficoll-Hypaque. Cells that did not rosette with sheep erythrocytes (E⁺) were placed in 24-well microtiter wells (10⁶ cells/ml) and cultured with 50 µl of EBV containing supernatant from the marmoset cell line. After approximately 3 weeks, EBV transformed cultures were coated with OKT4A (100 ng/10⁶ cells), washed and reacted with goat anti-mouse-IgG coated magnetic beads (40 beads/cell), according to the manufacturer's instructions (Advanced Magnetics, Cambridge, MA). Cells that bound beads were purified by magnetic sedimentation and placed back in culture. Magnetic bead selection with OKT4A was repeated at approximately 10 day intervals. After the first selection, approximately 30% of B cells bound OKT4A by FACS, after the second selection: 60–70% bound OKT4A and after three selections: 90% bound OKT4A. The cells were then cloned by limiting dilution in microtiter plates and screened by FACS for CD4 expression.

Immunoprecipitation

B lymphoblastoid cells were grown in methionine-free RPMI medium with 10% dialyzed FCS before a 1 hr pulse with ³⁵S methionine (10 µCi/10⁶ cells) and 1.5 hr chase with FCS and subsequent lysis in ice cold 10 mM TBS buffer containing 1% NP-40, 10 µg/ml iodoacetamide and 10 µg/ml phenylmethyl sulfonyl

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phosphate (PMSF). Lysates were cleared by a 30 min 14,000 rev/min spin in an eppendorf microfuge and affinity purified on a 4cc lentil lectin Sepharose 4B column (Pharmacia, Uppsala, Sweden). Eluate fractions (10⁶ cpm) were precleared first by reaction with a mixture of protein A-Sepharose (Pharmacia), Sepharose CL 4B (Pharmacia) and normal mouse serum. Next, the eluates were precleared by reaction with a mixture of these bead preparations and rabbit anti-mouse IgG. Aliquots of precleared eluates were then reacted with protein A-Sepharose beads (Pharmacia) coated with 5 µg of the following murine IgG2 mAb: OKT3, OKT4, OKT4A or W6/32. The beads were washed seven times in 0.3% NP-40, 10 mM Tris and 0.5 M NaCl before addition of SDS/2-Mercapto ethanol running solution and heated to 100°C for 5 min. Samples were separated in a 10% polyacrylamide gel. The gel was fixed in 30% methanol/10% glacial acetic acid for 15 hr and treated with Autofluor (National Diagnostics, Manville, NJ) for 1 hr. After drying the gel was used to expose XAR film (Kodak, Rochester, NY).

Oligodeoxynucleotide synthesis

Oligodeoxynucleotides (oligos) were synthesized by the phosphoramidite method on either a Cyclone Plus (MilliGen/Bioscience, Burlington, MA) or a Model 381A DNA Synthesizer (Applied Biosystems, Pasadena, CA), removed from the resin by treatment with concentrated NH₄OH followed by desalting on a NAP-5 column (Pharmacia) (for oligos <40 bases in length) with H₂O elution or by the use of an OPC column (Applied Biosystems) with 20% acetonitrile elution (for oligos >40 bases in length). The numbering used throughout this paper for CD4 nucleotides and amino acids corresponds to the Maddon numbering of the CD4 gene, RWHUT4 (Maddon *et al.*, 1985) with correction (Hussey *et al.*, 1988; Littman *et al.*, 1988).

RNA polymerase chain reaction

Total RNA was isolated from 10⁶ PHA stimulated E⁺ cells (Chirgwin *et al.*, 1979) and cDNA was prepared by reverse transcription of approximately 1.0 µg of total RNA using 200 units of moloney murine leukemia virus (MMLV) reverse transcriptase (Bethesda Research Labs (BRL), Bethesda, MD) for 30 min at 42°C in a reaction containing 10 pM of the primer, p1540-1517 (5' GAT CTG CTA CAT TCA TCT GGT CCG) in 20 µl of a buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT and 20 units of RNasin (Pharmacia). The reaction was heated to 95°C for 5 min to inactivate the enzyme. The first strand was amplified by PCR (Saiki *et al.*, 1985; Mullis *et al.*, 1986) under the following conditions: the initial template denaturing step (8 min at 94°C), followed by a 45-fold repetitive cycle of 2 min at 55°C (annealing), 2 min at 72°C (extension) and 2 min at 94°C (denaturation) using 2.5 units DNA Taq-polymerase (Perkin-Elmer Cetus,

Norwalk, CT), 200 µM each of dATP, dCTP, TTP and dGTP (Perkin-Elmer Cetus), and 50 pM of the primers p189-117 (5' GGC ACT TGC TTC TGG TGC TG) and p1540-1517 in a final volume of 100 µl PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin). After amplification the samples were analyzed by electrophoresis on a 1.0% agarose gel and were stained with ethidium bromide.

Construction of CD4^{OKT4+} plasmids by PCR recombination

A PCR strategy was employed to construct a CD4^{OKT4+} cDNA flanked by restriction sites, suited to expression in mammalian cells. We had extensive experience expressing immunoglobulin (Ig) genes using a vector derived from pD5 (Berkner and Sharp, 1985; see below) and a 5' gene segment derived from Ig that encodes an Ig signal sequence (Riechmann *et al.*, 1988). A computer-based algorithm, SIGSEQ2 (Daugherty *et al.*, 1990), predicted that cleavage of the Ig signal peptide fused to CD4 would result in a normal NH₂-terminus of mature CD4 (Hussey *et al.*, 1988). The Ig/CD4^{OKT4+} chimeric cDNA was generated by PCR construction of three overlapping DNA segments in separate PCR reactions, followed by assembly of the three segments into a full length cDNA using primers (amplifiers) that were complementary to the 5' and 3' termini of the full length molecule as described in Fig. 4 (Higuchi *et al.*, 1988).

The PCR product was digested with *Hind*III and *Xba*I and ligated into pSP72 (Promega) for sequencing. In order to express the OKT4⁺ allele, a *Hind*III/*Xba*I insert from these pSP72 constructs were ligated into pD5-tk-lyg which contained the following segments from pD5 (Berkner and Sharp, 1985): origin of adenovirus replication, the SV40 enhancer domain, the adenovirus major late promoter, the adenovirus 2 tripartite leader, a 5' splice donor from the adenovirus third leader and the SV40 late polyadenylation signal and in addition contained: an IgH enhancer, a 3' splice acceptor derived from an immunoglobulin locus, a multiple cloning site (containing *Hind*III and *Bam*HI) and a hygromycin resistance gene (Daugherty *et al.*, unpublished data).

Construction of CD4 plasmids

The *Eco*R1 fragment contains a full length cDNA insert from pMV7/T4 (Maddon *et al.*, 1985) encoding CD4 plus 5' and 3' untranslated sequence. The *Eco*R1 fragment was cloned into pCDNA-1 (Invitrogen) generating pCDNA-1/CD4^{OKT4+}.

The plasmid, pCDNA-1/CD4^{OKT4+} was generated by a three piece ligation of a 236 bp *Afl*II/*Sac*I (bp 370-606) fragment and a 265 bp *Sac*I/*Bst*EII (bp 606-871) fragment from pSP72/CD4^{OKT4+} into the pCDNA-1/CD4^{OKT4+} vector that had been digested with *Afl*II and *Bst*EII. Competent MC1061/p3 *E. coli*

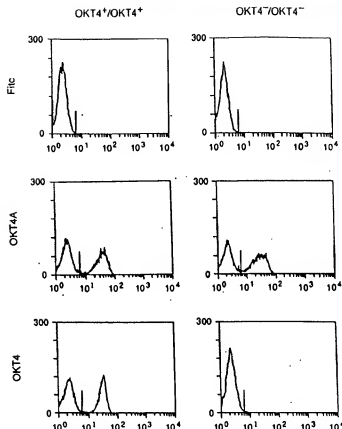


Fig. 1. FACS identification of a homozygous OKT4⁻ subject. The panels on the left are PBL from an individual with CD4^{OKT4+} molecules and on the right are from the individual with CD4^{OKT4-} molecules whose cells were subjected to the detailed biochemical analysis that follows. Shown are fluorescence cell histograms with the Y axis indicating number of cells, and the X axis showing relative fluorescence intensity. The mAbs used are indicated on along the Y axis. "Fic" designates that no primary antibody was added to cells and indicates the background fluorescence.

(Invitrogen) were transformed by the ligated DNA and grown on tetracycline (7.5 µg/ml) and ampicillin (12.5 µg/ml) plates.

Transfection of 293 cells

2×10^6 293 cells were plated on 100 mm Petri dishes 48 hr prior to transfection. The cells were fed with fresh medium 1 hr prior to transfection. Calcium phosphate precipitates were prepared (Graham and van der Eb, 1973; Pellicer *et al.*, 1978) using 20 µg of plasmid DNA per dish. After 15 hr at 37°C in 6% CO₂, the cells were fed with fresh media. Thirty-six hours after transfection, the cells were harvested by treating with trypsin-EDTA (Gibco, Grand Island, NY) for 30 sec and examined by FACS.

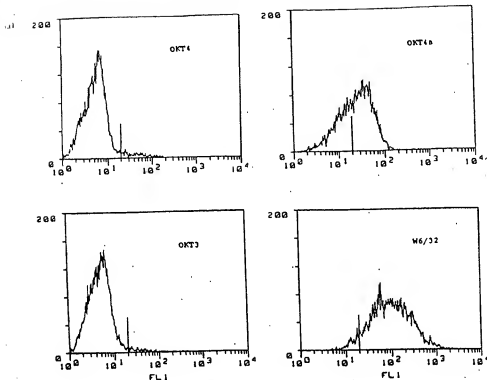
DNA sequencing

For sequencing of PCR products, the DNA product of RNA PCR described above was subjected to asymmetric PCR employing 50 pM of one primer and 0.5 pM of a second primer in a modification of the published protocol (Gyllenstein and Erlich, 1988).

Plasmid DNA was prepared by the alkaline lysis method (Birnbom and Doly, 1979). Annealing oligodeoxynucleotides were 17-mers based on the sequence of CD4^{OKT4+}. The sequencing reactions were performed according to the manufacturer's instructions (Sequenase, Version 2.0, U.S. Biochemical, Cleveland, OH).

RESULTS

In order to facilitate the characterization of the CD4^{OKT4-} protein and gene, B cells from a homozygous OKT4⁻ subject (Fig. 1) were immortalized with Epstein-Barr virus and subjected to positive selection using the mAb OKT4A and magnetic beads. Following antibody selection and limiting dilution cloning we isolated B lymphoblastoid cell clones expressing CD4^{OKT4-} (Fig. 2a). Immunoprecipitation of the CD4^{OKT4-} protein from CD4^{OKT4-} expressing B lymphoblastoid cells using the mAb OKT4A, revealed proteins that co-migrated with OKT4⁺ CD4 proteins on SDS-PAGE electrophoresis (Fig. 2b).



(b)

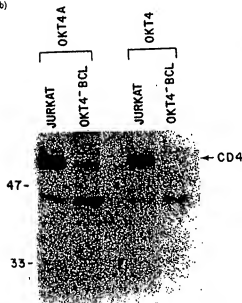


Fig. 2. Biochemical analysis of the CD4^{OKT4+} protein. (a) B cell lines which express CD4^{OKT4+}. Shown is the staining of a CD4⁺ B lymphoblastoid clone with the mAbs; OKT3 (anti-CD3), W6/32 (anti-Class I MHC) and two anti-CD4 mAbs, OKT4 and OKT4a. (b) Immunoprecipitation of CD4^{OKT4+}. Shown are radiographic exposures of radiolabeled proteins from a 30% CD4⁺ B cell line, immunoprecipitated with the mAbs, OKT4a and OKT4. As a control, radiolabeled lysate from Jurkat (which is OKT4⁺ by FACS) is also shown. The unidentified 45 kDa band bound to protein A-Sepharose beads independently of added mAb.

RNA analysis by hybridization with a CD4 probe demonstrated that the size of the message encoding the OKT4⁺ and OKT4⁺ alleles of CD4 were indistinguishable (not shown). Taken together, the protein and Northern data suggested that the CD4^{OKT4+} phenotype was not due to a large deletion in the CD4 gene.

In order to clone and sequence the cDNA encoding the CD4^{OKT4+} allele, poly(A⁺) RNA from PHA

stimulated peripheral T cells from the homozygous OKT4⁺ subject shown in Fig. 1, was amplified by RNA PCR using oligos anchored in the 5' and 3' untranslated (UT) sequences of the CD4 cDNA. Next, the structure of the CD4^{OKT4+} cDNA was mapped using oligos that amplified overlapping segments of the amplified CD4 template cDNA that contained the entire CD4 coding sequence. Each of the primer pairs amplified DNA segments that

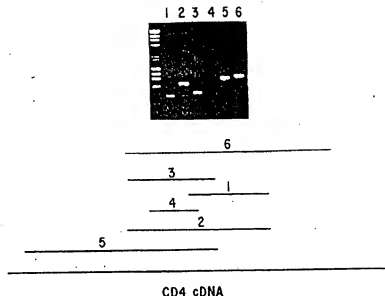


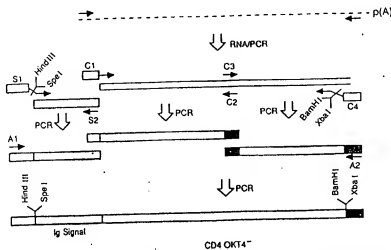
Fig. 3. Mapping the CD4^{OKT4}- cDNA by PCR. First strand cDNA from T cells with the OKT4⁻ phenotype was amplified by PCR using primer pairs based on the sequence of CD4^{OKT4} and the resulting PCR products were separated by electrophoresis in 1% agarose containing ethidium bromide and visualized by u.v. light. Lanes (1-6) represent the PCR products of the following primer pairs (the primers are identified by the nucleotide numbers represented in the primer in the 5' to 3' direction based on the nucleotide sequence of CD4 (1)). The sequences of the primers are: pr1190-1172 (5' AGC AGA CAC TGC CAC ATC), pr531-550 (5' GTG CAA TGT AGG AGT CCA AG), pr 950-929 (5' GCA TAC TGA GGC AAG GCC TG), pr617-634 (5' CCT GGA CAT GCA CTG TC), pr892-875 (5' TGG AGC TTA GGG TCC TG), pr97-117 (5' GGC ACT TGC TTC TGG TGC TG), pr1450-1430 (5' CAA ATG GGG CTA CAT GTC TTC) and pr832-849 (5' CCT TTG ACC TGA AGA AC). (1) pr332-849/pr1190-1173 (357 bp product), (2) pr352-551/pr1190-1173 (658 bp product), (3) pr352-551/pr950-931 (418 bp product); (4) pr617-633/pr893-877 (276 bp product); (5) pr98-117/pr950-931 (852 bp product); and (6) pr352-551/pr1451-1431 (919 bp product). The DNA standards are λ /HindIII (measuring 23, 9, 6.6, 4.4, 2.3 and 2.0 kb) and ϕ /HaeIII (measuring 1.3, 1.1, 0.9, 0.6 and 0.3 kb) (BRL).

corresponded to the expected sizes for the OKT4⁺ allele (Fig. 3) demonstrating that the OKT4⁻ allele did not contain a large deletion of nucleotides, relative to the OKT4⁺ allele.

The RNA PCR products were then partially sequenced and complete sequence homology was observed between the OKT4⁺ and OKT4⁻ alleles in several regions including the 5' and 3' UT regions as well as the nucleotides encoding the NH₂- and COOH-terminal amino acids of the mature proteins. In addition, the nucleotide sequence including nucleotides 877-894, which represents an internal region, approximately midway through the coding region, was also identical. Given these regions of nucleotide sequence identity between the OKT4⁺ and OKT4⁻ alleles, a PCR strategy was employed to clone a gene encoding CD4^{OKT4}- (Fig. 4). The PCR strategy was designed to fuse an Ig signal sequence onto a gene encoding the mature CD4 protein in order to facilitate screening of clones for expression of the phenotype (Fig. 4). Two separate PCR reactions amplified the 5' and 3' halves of the coding region of CD4^{OKT4}- cDNA from the template of the RNA PCR product described above (Fig. 4). The resulting PCR product was subcloned in pSP72

(Promega) and four clones that were found to contain the full length recombinant, CD4 cDNA were completely sequenced.

The pSP72/CD4^{OKT4}- clones revealed two consistent nucleotide substitutions in four out of four clones analyzed: a C \rightarrow T transition at nucleotide 352 that is silent and another C \rightarrow T transition at nucleotide 867 that results in an arginine \rightarrow tryptophan substitution at amino acid residue 240. Each clone contained at least two additional nucleotide substitutions, relative to the OKT4⁺ sequence, that were not present in the other three clones or in the RNA PCR product sequenced by asymmetric PCR. These additional mutations appeared to represent random mutations introduced during the PCR amplification using the taq polymerase. Several clones were screened for expression by ligation into the pD5-1k-hygro vector and transfection into 293 cells. One clone expressed CD4^{OKT4}- molecules by FACS analysis (not shown). This clone contained 867 C \rightarrow T, but also contained an additional mutation (coding for an amino acid substitution in the V4 domain of the CD4 protein) that was not present in the other clones or in the sequence obtained from direct sequencing of the RNA. In order to test the hypothesis that the

[illegible]

single, arginine \rightarrow tryptophan substitution (867 C \rightarrow T) encoded the OKT4⁻ phenotype, a full length cDNA containing only the 867 C \rightarrow T mutation, on the background of cDNA^{OKT4⁺}, was generated by ligation of an internal *AFII-BstEI* fragment (bp 307–871) from pSP72/cDNA^{OKT4⁺} into pCDNA1/cDNA^{OKT4⁺} (which includes the native CD4 signal sequence). In transient expression assays, this cDNA directed the expression of CD4 molecules with the CD4^{OKT4⁻} phenotype (Fig. 5a). The fact that this cDNA (pCDNA1/cDNA^{OKT4⁻}) expressed a CD4^{OKT4⁻} protein, confirmed that a single amino acid substitution of arginine for tryptophan at residue 240 in CD4 accounts for the OKT4⁻ phenotype (Fig. 5b).

In this study we have precisely characterized the molecular basis for a common African allele of the human CD4 molecule. The allele is phenotypically characterized by the absence of reactivity of CD4 molecules with the mAb OKT-4 (Bach *et al.*, 1981; Fuller *et al.*, 1984; Karol *et al.*, 1984; Azozia *et al.*, 1985; Saio *et al.*, 1984; Stohl and Kunkel, 1984). Of African-Americans studied, 20.2% express this allele as heterozygotes and 8.2% express this allele as homozygotes (Fuller *et al.*, 1984). The present study demonstrates that the genetic basis for this phenotype in one homozygous individual is a single amino acid

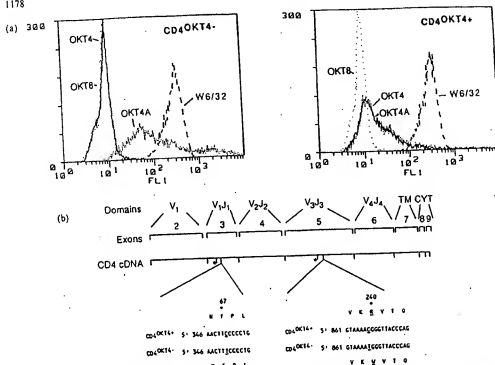


Fig. 5. A single amino acid substitution (R→W 240) encodes the CD4^{OKT4-} phenotype. (a) Expression of pCDNA-1/CD4^{OKT4-} in 293 cells. Shown are fluorescence cell histograms of 5×10^5 293 cells transfected with pCDNA-1/CD4^{OKT4-} (top panel left) or pCDNA-1/CD4^{OKT4+} (top panel right) and stained with the indicated mAbs. (b) Physical map of CD4^{OKT4-} cDNA showing relationship of nucleotide substitutions to coding changes, and exon and domain structures. Asterisks indicate potential other CD4 polymorphisms, Trp→Arg (62) and Phe→Ser (229), previously published (Fisher *et al.*, 88).

substitution of tryptophan for arginine at amino acid residue 240 in the extracellular V3 domain of CD4.

Comparison of the sequence surrounding CD4 amino acid 240 with the CD4 sequence from other species, indicates that a positively charged amino acid (either arginine or lysine) is present at this position in diverse species in the primate (Camerini and Seed, 1990) and rodent (Maddon *et al.*, 1987; Clark *et al.*, 1987) orders (Fig. 6). In rat and mouse, arginine 240 is represented by a lysine that conserves the positively charged amino acid (Fig. 6). The fact that the arginine→tryptophan substitution at residue 240 does not maintain a positively charged residue at this

position, suggests that a functionally important consequence may be associated with the OKT4⁻ phenotype. However, the fact that human and other primate CD4 encodes a lysine at the nearby residue 239, may indicate redundancy in the positively charged amino acids in this region, mollifying the effects of the OKT4⁻ substitution (Fig. 6).

Although the *in vivo* functional consequences, if any, of the CD4^{OKT4-} phenotype remains largely unexplored, it is of interest that some OKT4⁻ individuals with the autoimmune disease, SLE, as well as their healthy (non-SLE) OKT4⁺ relatives, demonstrate abnormal interactions between T and B cells

		240	
		W	
huOKT4-		LKNKEVSVKR	VTQDKPLQMG KKLPL
human	(225)	SWITFD
rhesus	(225)
chimpanzee	(225)
mouse	(224)	P..S.S I.....	..QK S.K.L..LQ ET..
rat	(224)S S...QK...QK S.SNP.F.LS ET..	
poly (Ig) R	(157)	--LI.I SSS..AKDP. YKGRIT..IQ STTAK	

Fig. 6. Comparison of the CD4 sequence in the region of the OKT4⁻ mutation with other related proteins. The human sequence in this region is completely conserved between chimpanzee, rhesus macaque (Camerini and Seed, 1990). The mouse (Maddon *et al.*, 1987) and rat (Clark *et al.*, 1987) CD4 proteins have a lysine at the position equivalent to human residue 240. The comparison of the CD4 sequence to that of the rat poly Ig receptor is published (Clark *et al.*, 1987) and indicates that the poly Ig receptor contains an arginine at the residue equivalent to CD4 240. Dots indicate identity with the human sequence and dashes represent gaps in the amino acid sequence used to maximize homology (Clark *et al.*, 1987).

in the generation of CD4^{OKT4-} phenotype. Because binding regions of the mutation, s in T-B interaction likely be a consequence to Ia binding. In CD4 may physically interact with (Saizawa *et al.*, 1989; Riv. The molecular t unknown but it arginine to trypt proximal, V3 d the molecule th ligand confined cell receptor. f OKT4⁻ allele context of a f CD4⁺ T cell el in detail.

We and oth binds to rece *et al.*, 1990; L CD4 that bin provided evid IgG interacti- region of the 1990). Altho cellular CD4 lished), and CD4-IgG bi may bind to substitution important i a domain th arginine at arginine at there is no IgG molecu- lation actions is (Nicholsor Binding tested oth these allel example, : subject th OKT4C1 recently i mAb Lev bind to a (Jameso nucleoti mutations v a clone (Fisher phisms

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the generation of antibody responses *in vitro* (Stohl *et al.*, 1985). Because the mutation accounting for the CD4^{OKT4⁺} phenotype resides in V3 and not in the 14 amino acid regions of CD4, any functional consequences of the mutation, such as the reported abnormalities in T-B interactions (Stohl *et al.*, 1985), would most likely be a consequence of CD4 functions unrelated to binding. In this regard, there is evidence that CD4 may physically interact in a functionally important manner with the antigen specific T cell receptor (Saizawa *et al.*, 1987; Anderson *et al.*, 1988; Rojo *et al.*, 1989; Rivas *et al.*, 1988; Mittler *et al.*, 1989). The molecular basis for this putative interaction is unknown but it is of interest that the location of the arginine to tryptophan substitution in the membrane proximal, V3 domain of CD4, suggests a region of the molecule that might interact horizontally with a ligand confined to the same membrane, such as the T cell receptor. Efforts are underway to express the OKT4⁺ allele as well as the OKT4⁻ allele, in the context of a functional TCR on the surface of a CD4⁺ T cell clone in order to investigate this point in detail.

We and others have previously reported that IgG binds to recombinant truncated CD4 (Lederman *et al.*, 1990; Lenert *et al.*, 1990). The precise site on CD4 that binds IgG is controversial, but we have provided evidence suggesting that at least one site of IgG interaction with CD4 is either the V3 or V4 region of the soluble CD4 molecule (Lederman *et al.*, 1990). Although we cannot detect IgG binding to cellular CD4 molecules (Lederman *et al.*, unpublished), and the physiological significance of CD4-IgG binding is not known, the fact that IgG may bind to a site near to the present amino acid substitution is interesting. In this regard, it may be important to note that the poly Ig receptor contains a domain that is homologous to CD4 V3 and has an arginine at the amino acid residue analogous to the arginine at 240 of CD4 (Clark *et al.*, 1987). Although there is no direct evidence that T cells interact with IgG molecules during T-B collaboration, the observation that aggregated IgG inhibited T-B interactions is consistent with such an interaction (Nicholson and McDougall, 1981).

Binding studies using anti-CD4 mAbs have detected other polymorphisms, but the incidence of these alleles has not been studied in populations. For example, a polymorphism was detected in an OKT4⁺ subject that was deficient in the binding of the mAb OKT4C (Bach *et al.*, 1981). In addition, a subject was recently reported to be deficient in the binding of the mAb Leu3a (Angadi, 1990). Both OKT4C and Leu3a bind to amino acid residues in the V1 domain of CD4 (Jameson *et al.*, 1988). Furthermore, two other nucleotide changes that encode amino acid substitutions were identified by the independent isolation of a clone encoding CD4 from a T cell cDNA library (Fisher *et al.*, 1988). Interestingly, these polymorphisms are encoded by sites on the CD4 gene close to

the positions where nucleotide changes are found in the CD4^{OKT4⁺} allele analyzed in the present work (Fig. 6). In that study no correlation was made between the genetic structure and the phenotype of the encoded protein in relation to the binding of mAbs. Therefore, it appears that several polymorphic alleles of CD4 exist and the limited genetic data suggest that mutations are clustered into two areas of the protein, encoded by different exons (Fig. 5b) (Maddon *et al.*, 1987). It is not clear if these clusters indicate areas of active mutation or if there are selective functional pressures exerted on these regions. In this regard, it is interesting that analysis of the CD4 protein sequence suggests that amino acids 62, 229 and 240 may be located on alpha helices (Maddon *et al.*, 1987) which often form turns on the external surface of the protein.

It is interesting to note that a high prevalence of the OKT4⁺ allele coexists in Africa with a relatively high prevalence of human infection by the CD4 tropic pathogenic human retroviruses (HIV-1 and -2). The infectibility of CD4^{OKT4⁺} cells by HIV in culture is established (Hoxie *et al.*, 1986), but a potentially important avenue of future study is to determine if the CD4 polymorphism characterized in the present study affects the clinical outcome of HIV infection. If this were the case, molecular analysis of the mechanism of such an effect would be potentiated by the genetic tools described here.

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Exhibit 4

β -Endorphin omission analogs: Dissociation of immunoreactivity from other biological activities

(peptide synthesis/radioimmunoassay/analgesia/ileal opiate activity/opiate receptor-binding activity)

CHOH HAO LI*, DONALD YAMASHIRO*, LIANG-FU TSENG†, WEN-CHANG CHANG*, AND PASCUAL FERRARA*

*Hormone Research Laboratory, University of California, San Francisco, California 94143, and †Department of Pharmacology, The Medical College of Wisconsin, Milwaukee, Wisconsin 53233

Contributed by Choh Hao Li, March 5, 1980

ABSTRACT An analog of human β -endorphin with omission of four residues at positions 11, 14, 20, and 22 has been synthesized. This analog and other synthetic analogs with deletion of a single amino acid at position 2, 5, 6, 10, 11, 12, 13, 15, or 22 have been assayed for analgesic potency, ileal opiate activity, opiate receptor-binding activity, and immunoreactivity. Results show that deletion of a single amino acid of the β -endorphin molecule outside of the enkephalin segment to give des-Gln¹¹, des-Thr¹⁴, des-Pro¹⁵, des-Leu¹⁴, des-Val¹⁸, des-Asn²⁰, or des-Ile²² β -endorphin markedly reduced or abolished the immunoreactivity yet gave substantial retention of opiate potencies. Deletion of a single amino acid of β -endorphin within the enkephalin segment (des-Gly⁵ or des-Met⁶ β -endorphin) did not markedly affect the immunoreactivity; however, the opiate activities were abolished or markedly reduced. The data indicate a clear dissociation of immunoreactivity from analgesic, ileal-opiate, and opiate receptor-binding activities.

β -Endorphin (β -EP) (ref. 1; see Fig. 1) is a naturally occurring opioid peptide with potent opiate analgesic activity after intracerebral (2) or intravenous injections (3, 4). Studies on structure-activity relationships indicate that the entire β -EP molecule is necessary for full analgesic potency (5). In addition, omission of a single amino acid residue at position 14 or 20 abolishes immunoreactivity yet gives retention of opiate potency (6). We present herein biological activities of synthetic analogs with deletion of a single amino acid at position 2, 5, 6, 10, 11, 12, 13, 15, or 22 as well as a synthetic analog with omission of four residues at positions 11, 14, 20, and 22. The analogs were assayed for analgesic activity by the tail-flick test, ileal opiate activity by the guinea pig ileum method, opiate receptor-binding activity by displacement of [³H]- β -EP binding to membrane of rat brain, and immunoreactivity by radioimmunoassay. Results show a clear dissociation of immunoreactivity from other biological activities.

MATERIALS AND METHODS

Synthesis of single-deletion analogs of β -EP has been described (6, 7). Des-Gln¹¹, Leu¹⁴, Asn²⁰, Ile²² β -EP was synthesized by the solid-phase method (8). It was performed with Boc(Bzl)Glu brominated polymer (0.34 mmol/g) (4) on a Beckman model 990 peptide synthesizer. A fully automated symmetrical anhydride program (5) was used except for the Asn residue, which was incorporated by procedures described for the synthesis of β -EP (4). The following amino acid residues in the β -EP sequence were omitted in the synthesis: Gln¹¹, Leu¹⁴, Asn²⁰, and Ile²². From 295 mg (100 μ mol) starting resin there was ob-

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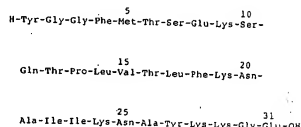


FIG. 1. Amino acid sequence of β -EP (β -EP: His-27, Gln-31). Residues 1-5 correspond to [Met]enkephalin.

tained, after removal of the last Boc group, 768 mg of protected peptide resin corresponding to des-Gln¹¹, Leu¹⁴, Asn²⁰, Ile²² β -EP. Cleavage and deprotection in HF (9), gel filtration on Sephadex C-10 (0.5 M acetic acid), and chromatography on carboxymethylcellulose were performed as described (4). From additivity rules for the hydrophobicities of amino acid residues in β -EP (7) it could be predicted that partition chromatography on Sephadex C-50 in the same solvent system used for β -EP (4) would give an R_F of 0.27 based on the reported R_F of 0.40 for β -EP. The experimental value of R_F was found to be 0.26. The overall yield of des-Gln¹¹, Leu¹⁴, Asn²⁰, Ile²² β -EP based on 50 μ mol of starting resin was 39.0 mg (26%). The product (50- μ g samples) was homogeneous on thin-layer chromatography on silica gel in 1-butanol/pyridine/acetic acid/H₂O (5.5:1.4, vol/vol), R_F 0.40 (ninhydrin and chloramine-toluidine detection), and in paper electrophoresis on Whatman 3 MM at pH 3.7 (R_F 0.58 relative to Lys) and pH 8.7 (R_F 0.45 relative to Lys) at 400 V (5 hr, ninhydrin detection). Amino acid analysis of a 24-hr HCl hydrolysate gave (theoretical values in parentheses): Lys, 4.91 (5); Asp, 1.06 (1); Thr, 3.12 (3); Ser, 1.92 (2); Glu, 2.10 (2); Pro, 0.96 (1); Gly, 2.97 (3); Ala, 2.12 (2); Val, 1.02 (1); Met, 0.98 (1); Ile, 1.00 (1); Leu, 1.07 (1); Tyr, 1.96 (2); Phe, 1.96 (2). Amino acid analysis of an enzymic digest (trypsin and chymotrypsin followed by leucine aminopeptidase) gave: Lys, 4.90 (5); Thr + Ser + Asn, 6.30 (6); Glu, 1.96 (2); Pro, 0.91 (1); Gly, 2.84 (3); Ala, 2.14 (2); Val, 1.12 (1); Met, 0.98 (1); Ile, 1.06 (1); Leu, 1.10 (1); Tyr, 1.90 (2); Phe, 1.84 (2).

Opiate activities were assessed both *in vitro* and *in vivo*. The ileal opiate activity *in vitro* was measured by the inhibition of electrically stimulated contraction of guinea pig ileum preparation (10), and the opiate receptor-binding assay was performed according to the procedure recently described (11, 12), using [³H]-Tyr²⁷ β -EP (13) as the primary ligand and synthetic

Abbreviations: β -EP, β -endorphin (subscripts 1 and 2 indicate β -EP from human and camel pituitaries); IC₅₀, 50% inhibitory concentration.

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Table 1. Ileal opiate activities of omission analogs of β -EP

Synthetic peptide	IC ₅₀ , ^a nM	Relative potency
β -EP	91	100
Des-Gly ² - β -EP	9100	1
Des-Leu ¹⁴ - β -EP	65	140
β -EP	58	100
Des-Thr ⁶ - β -EP	60	97
Des-Ser ¹⁰ - β -EP	49	118
Des-Thr ¹² - β -EP	50	116
β -EP	132	100
Des-Met ⁵ - β -EP	2000	7
Des-Val ¹⁵ - β -EP	107	123
Des-Ile ²² - β -EP	67	197
β -EP	22	100
β -EP	22	100
Des-Gln ¹¹ - β -EP	22	100
Des-Pro ¹³ - β -EP	20	110
Des-Asn ²⁰ - β -EP	20	110
Des-Gln ¹¹ -Leu ¹⁴ -Asn ²⁰ -Ile ²² - β -EP	16	138

^a Guinea pig ileum assay; IC₅₀ is the concentration that gives 50% inhibition of contraction.

β -EP (14) or β -EP (4) as standard competing ligand. The analgesic activity *in vivo* was assessed in mice by the tail-flick method (15) as described (5). Radioimmunoassay was carried out by the procedure described (16, 17).

RESULTS

The opiate activities *in vitro* of various omission analogs as assayed by the guinea pig ileum preparation are summarized in Table 1. Deletion of Gly² or Met⁵ in the [Met]enkephalin segment of β -EP causes a marked decrease of opiate potency. On the other hand, deletion of a single amino acid residue outside the [Met]enkephalin segment does not alter the opiate potency and in some cases even enhances it. For example, des-Ile²² and des-Leu¹⁴- β -EP are 1.4 and 1.97 times as potent as the intact peptide, respectively. Omission of four residues in positions 11, 14, 20, and 22 increases the potency to 138% compared with β -EP.

Table 2. Analgesic potencies of omission analogs of β -EP

Synthetic peptide	AD ₅₀ , ^a nmol/mouse	Relative potency
β -EP	0.026 (0.020-0.032)	100
Des-Gly ² - β -EP	>25	<0.1
Des-Gln ¹¹ - β -EP	0.033 (0.021-0.048)	79
Des-Pro ¹³ - β -EP	0.113 (0.089-0.149)	23
Des-Asn ²⁰ - β -EP	0.057 (0.042-0.075)	46
β -EP	0.043 (0.035-0.075)	100
Des-Met ⁵ - β -EP	0.219 (0.099-0.487)	20
Des-Thr ⁶ - β -EP	0.059 (0.048-0.077)	73
Des-Ser ¹⁰ - β -EP	0.047 (0.027-0.092)	92
Des-Thr ¹² - β -EP	0.045 (0.024-0.095)	96
Des-Leu ¹⁴ - β -EP	0.057 (0.033-0.093)	75
Des-Val ¹⁵ - β -EP	0.179 (0.131-0.241)	24
Des-Ile ²² - β -EP	0.075 (0.046-0.117)	57
β -EP	0.064 (0.026-0.17)	100
Des-Gln ¹¹ -Leu ¹⁴ -Asn ²⁰ -Ile ²² - β -EP	0.99 (0.46-2.19)	7

^a Median antinociceptive dose (95% confidence limit).

Table 3. Opiate receptor-binding activities of omission analogs of β -EP

Synthetic peptide	IC ₅₀ , pM	Relative potency
β -EP	250	100
Des-Gly ² - β -EP	50,000	0.5
Des-Met ⁵ - β -EP	12,000	2
Des-Thr ⁶ - β -EP	550	45
Des-Ser ¹⁰ - β -EP	280	90
Des-Gln ¹¹ - β -EP	210	120
Des-Thr ¹² - β -EP	270	93
Des-Pro ¹³ - β -EP	860	29
Des-Leu ¹⁴ - β -EP	430	58
Des-Val ¹⁵ - β -EP	390	64
Des-Asn ²⁰ - β -EP	530	47
Des-Ile ²² - β -EP	180	139
β -EP	560	100
β -EP	250	224*
Des-Gln ¹¹ -Leu ¹⁴ -Asn ²⁰ -Ile ²² - β -EP	610	92*, 41*

* Relative to β -EP.

† Relative to β -EP.

Table 2 presents the analgesic potencies *in vivo* of the synthetic analogs. Des-Gly²- β -EP is less than 0.1% as potent as β -EP, and des-Met⁵- β -EP exhibits only 20% potency relative to that of β -EP. The majority of analogs with deletion of a single amino acid residue outside the [Met]enkephalin segment retain substantial analgesic potency. However, deletion of four residues (positions 11, 14, 20, and 22) drops potency to 7% of that of the intact molecule.

As in the ileal opiate activity assay, the deletion of a single amino acid residue at position 2 or 5 markedly reduced the potency in the opiate receptor assay using membranes of rat brain. Deletion of a single amino acid residue outside the enkephalin segment, however, did not markedly alter the opiate receptor-binding potency (Table 3). Even deletion of four residues simultaneously had no drastic effects.

Table 4 summarizes the immunoreactivity of omission analogs by the β -EP radioimmunoassay system. Deletion of a single amino acid residue at position 11, 12, 13, 14, 15, or 20 abolished or markedly reduced the abilities of these peptides

Table 4. Immunoactivity of omission analogs of β -EP

Synthetic peptide	IC ₅₀ , ^a pM	Relative activity
β -EP	72	100
Des-Gln ¹¹ - β -EP	800	9
Des-Pro ¹³ - β -EP	1,029	7
Des-Leu ¹⁴ - β -EP	>10,000	<1
Des-Val ¹⁵ - β -EP	>10,000	<1
Des-Asn ²⁰ - β -EP	>10,000	<1
Des-Ile ²² - β -EP	248	29
β -EP	56	100
Des-Gly ² - β -EP	88	82
β -EP	51	100
β -EP	51	100
Des-Met ⁵ - β -EP	67	76
Des-Thr ⁶ - β -EP	48	108
Des-Ser ¹⁰ - β -EP	70	73
Des-Thr ¹² - β -EP	1,300	3
Des-Gln ¹¹ -Leu ¹⁴ -Asn ²⁰ -Ile ²² - β -EP	>10,000	<1

* Radioimmunoassay.

to bind to the antibodies of β -EP. On the other hand, omission of Gly² or Met⁵ in the [Met]enkephalin segment of β -EP as well as Thr⁶ or Ser¹⁰ resulted in retention of high immunoreactive potency.

DISCUSSION

Previous studies indicated the importance of the Tyr¹, Phe⁴, and Met⁵ residues for the production of opiate analgesic activity (18–20). In this study, we found that deletion of Gly² or Met⁵ in the [Met]enkephalin segment of β -EP drastically lowers opiate analgesic, ileal opiate, and receptor-binding potency, whereas nearly full immunoreactivity is retained. Of these two residues, Gly² appears to be more important for the production of opiate activities. Omission of a single residue outside of this segment does not cause considerable loss of biological activities, but immunoreactivity is markedly affected. Omission of a single amino acid at position 14, 15, or 20 abolishes immunoreactivity yet retains significant amounts of other biological activities. This indicates that the active sites in the β -EP molecule for binding to the β -EP antibodies resides in positions 11 to 22. Thus we have discovered an instance in which deletion of a single amino acid residue in a biologically active peptide abolishes immunoreactivity.

Des-Gln¹¹, Leu¹⁴, Asn²⁰, Ile²², β -EP has virtually no immunoreactivity and exhibits somewhat higher ileal opiate and significant receptor-binding activity in comparison to β -EP. Analgesic potency of this analog is only 7% when compared with the activity for the intact molecule. In an earlier report (16), a lack of correlation between immunoreactivity and opiate activity as assayed by the guinea pig ileum preparation has been noted.

When the relative ileal opiate activities for des-Gln¹¹, des-Leu¹⁴, des-Asn²⁰, and des-Ile²², β -EP are averaged, a value of 137 is obtained. It is interesting that the observed opiate activity of des-Gln¹¹, Leu¹⁴, Asn²⁰, Ile²², β -EP is 138. On the other hand, the similarly calculated values for the other activities of des-Gln¹¹, Leu¹⁴, Asn²⁰, Ile²², β -EP (receptor-binding activity, 124; analgesic potency, 64, and immunoreactivity, 10) diverge increasingly from the experimental data in the order given. These results illustrate the insensitivity of the ileal assay to such structural alterations, while the other assays show sensitivity in the order immunoreactivity > analgesic activity > receptor-binding activity.

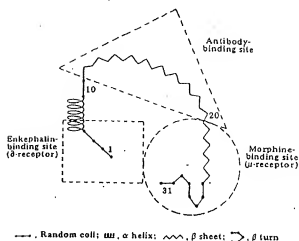


FIG. 2. Proposed binding sites in the primary structure of β -EP. Predicted secondary structure of β -EP was taken from ref. 23.

The data, summarized in Table 5, clearly show the dissociation of immunoreactivity from analgesic, ileal opiate, and receptor-binding activities. Moreover, there is a fair correlation between analgesic potency and receptor-binding activity if des-Gln¹¹, Leu¹⁴, Asn²⁰, Ile²², β -EP is excluded. This omission analog possesses significant receptor-binding activity and low analgesic potency. The lack of correlation between opiate receptor-binding activity and analgesic potency has recently been observed with synthetic analogs with extension at the COOH terminus (21). These data emphasize again the importance of not relying on a single assay procedure for the characterization of biologically active peptides.

There are at least two receptors for opioid peptides in the brain (22): the μ receptors for morphine and the δ receptors for the enkephalins. The data presented herein, together with the recent findings that β -EP-(6–31) and β -EP-(20–31) segments inhibit morphine-induced analgesia (unpublished), suggest the presence of three binding sites in the β -EP molecule as shown in Fig. 2. The first site resides in the [Met]enkephalin segment [enkephalin-binding site ("delta-receptor")] and the second consists

Table 5. Relative biological activities of omission analogs of β -EP.

Synthetic peptide	Analgesic potency	Opiate activity	Receptor-binding activity	Immunoreactivity
β -EP	100	100	100	100
Des-Gly ² , β -EP	<0.01	1	0.6	82
Des-Met ⁵ , β -EP	20	7	2	76
Des-Thr ⁶ , β -EP	73	97	45	106
Des-Ser ¹⁰ , β -EP	92	118	90	73
Des-Gln ¹¹ , β -EP	79	100	120	9
Des-Thr ¹² , β -EP	96	116	93	3
Des-Pro ¹³ , β -EP	23	110	29	7
Des-Leu ¹⁴ , β -EP	75	140	58	<1
Des-Val ¹⁵ , β -EP	24	123	64	<1
Des-Asn ²⁰ , β -EP	46	110	47	<1
Des-Ile ²² , β -EP	57	197	139	29
β -EP	100	100	100	100
β -EP	100	100	224*	100
Des-Gln ¹¹ , Leu ¹⁴ , Asn ²⁰ , Ile ²² , β -EP	7	138	92*, 41†	<1

* Relative to β -EP.

† Relative to β -EP.

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of the COOH-terminal segment [β -EP-(21-31)] [morphine-binding site ("μ-receptor")]. The middle segment [β -EP-(11-20)] is the antibody-binding site. Studies on the *in vivo* and *in vitro* biological profiles of synthetic β -EP analogs may possibly clarify the role of these binding sites.

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